The adaptive regulation of amino acid transport system A is associated to changes in ATA2 expression

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Abstract The activity of transport system A for neutral amino acids is adaptively stimulated upon amino acid starvation. In cultured human fibroblasts this treatment causes an increase in the expression of the ATA2 system A transporter gene. ATA2 mRNA increase and transport stimulation are suppressed by system A substrates, but they are unaffected by other amino acids. Supplementation of amino acid-starved cells with substrates of system A causes a decrease in both ATA2 mRNA and system A transport activity. These results suggest a direct relationship between ATA2 expression and system A transport activity.

Key words: Amino acid transport; Proline; Human fibroblast; Transport regulation; Amino acid starvation

1. Introduction

System A is a heavily regulated, secondarily active, sodium-dependent transport mechanism for neutral amino acids widely expressed in mammalian tissues. The system was operationally identified because it tolerates *N*-alkylated substrates [1]. However, a cDNA-encoding system A transporter has been cloned only very recently [2–5]. This isoform, designated ATA2 (also known as SAT2 and SA1), is widely expressed in rat and human tissues [2–5].

The prolonged incubation of cultured human fibroblasts [6], as well as of many other cell models (see [7] for review), in amino acid-free saline solution causes a slow, protein synthesis-dependent stimulation of system A transport activity. Conversely, the restoration of extracellular amino acid availability provokes a decrease of transport activity. The molecular mechanisms underlying these transport changes, collectively named adaptive regulation [8], have remained elusive thus far.

Employing cultured human fibroblasts, we demonstrate here that the availability of extracellular amino acids markedly affects ATA2 mRNA, whose abundance changes in parallel with system A transport activity. These results indicate that system A adaptive regulation is associated with changes in the expression of the transporter gene.

2. Materials and methods

2.1. Cell lines and incubations

Cultured human fibroblasts were grown, as described previously [9], in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The experimental protocol employed to trigger the adaptive enhancement of system A transport activity [6] consisted in starving cells of amino acids through a prolonged incubation in Earle's balanced salt solution (EBSS) supplemented with 10% dialyzed FBS.

2.2. Transport studies

For the experiments cells were seeded on 24-well trays (Nunc). Transport activity of system A was determined, as described previously [6,10], from the initial influx of L-proline, which behaves as a site A-specific substrate in human fibroblasts [11]. The amino acid substrate was employed at a concentration of 0.1 mM in 1-min uptake assays at 37°C. Under these conditions the amino acid influx is linear for more than 10 min (unpublished results). Transport activity is expressed as nmol/mg protein/min. Statistical significance was assessed through ANOVA.

2.3. Northern analysis

Preliminary experiments, performed through cloning of cDNA fragments of ATA and subsequent sequencing (not shown), demonstrated that human fibroblasts express hATA2. Total RNA was extracted from confluent fibroblasts, incubated under the experimental conditions described for each experiment, with the guanidine thiocyanate/cesium chloride method [12]. For Northern analysis 15 μg of RNA per lane was subjected to electrophoresis under denaturing conditions on 1% agarose gel containing 2.2 M formaldehyde. After staining with ethidium bromide, the total RNA was transferred overnight onto positively charged nylon membranes (Schleicher and Schuell SpA, Italy). Northern blots were hybridized overnight at 42°C in 50% formamide to ^{32}P-labeled full-length cDNA probes (Ready to Go DNA Labeling Beads, Pharmacia) encoding human ATA2 [2,3] and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), kindly provided by Dr. R. Allen [13]. The relative abundance of ATA2 mRNA was expressed according to the following formula:

$$[(a/b)\times 100] : (c/d) \quad (1)$$

where *a*, *b*, *c*, and *d* are the densitometric values (expressed in arbitrary units) of, respectively, ATA2 mRNA band under the experimental condition, GAPDH mRNA band under the experimental condition, ATA2 mRNA band under control conditions, and GAPDH mRNA band under control conditions.

2.4. Materials

Serum and culture medium were obtained from Gibco. L-[5-^{3}H]-Proline (32 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were pur-

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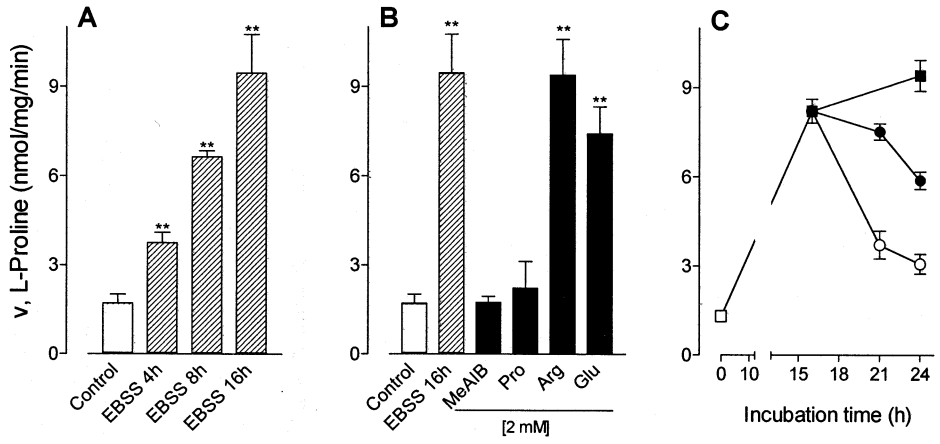

Fig. 1. Adaptive regulation of system A transport activity. A: Transport of L-proline (0.1 mM, 2 μCi/ml, uptake time 1 min) was measured, as described in Section 2, in cultured human fibroblasts preincubated for the indicated times in EBSS+10% dialyzed FBS (cross-hatched bars). In control cells transport was determined without any preincubation in EBSS (open bar). B: Transport of L-proline was determined after 16 h of incubation in EBSS+10% dialyzed FBS (cross-hatched bar) or in the same solution supplemented with the indicated amino acids at a concentration of 2 mM (solid bars). In control cells transport was determined without any preincubation in EBSS (open bar). C: Cells were incubated for 15 h in EBSS+10% dialyzed FBS (■). After this period, the incubation was prolonged in the same solution (■), or in EBSS supplemented with 5 mM L-proline (●) or in complete D-MEM (○). Control cells (no preincubation, □). In all cases points represent the mean of four independent determinations with S.D. indicated when greater than the size of the point. The experiment was repeated three times with comparable results. $^{**}P < 0.01$ vs. control (no preincubation).

chased from DuPont de Nemours. Sigma was the source of the other chemicals.

3. Results

The incubation of cultured human fibroblasts in amino acid-free EBSS stimulated system A transport activity (Fig. 1A). The transport of L-proline, mostly due to system A operation in human fibroblasts [11], was already significantly higher than control after 4 h of incubation under amino acid-free conditions. If starvation was prolonged, transport activity steadily increased up to at least 16 h. At this time, proline uptake was more than five-fold stimulated compared to the control value. The presence of a single substrate of system A, such as L-proline or the non-metabolizable analogue MeAIB, employed at a concentration of 2 mM, completely prevented the stimulation of system A transport activity (Fig. 1B). Conversely, the starvation-induced transport stimulation was not affected by the presence of glutamate or arginine, amino acids that are not substrates of system A [14,15]. If proline was added to EBSS at later times of amino acid starvation (Fig. 1C), the transport activity of system A decreased progressively. A steeper fall of proline transport was observed if the amino acid-free extracellular medium was replaced with complete DMEM. After 9 h of extracellular amino acid restitution, transport activity was lowered by more than 60% compared with high-transporting, starved cells, although it was still significantly higher than control values obtained at the beginning of the experiment.

High stringency Northern blot analysis of ATA2 expression in cultured human fibroblasts is shown in Fig. 2. Maintained in complete growth medium (control conditions) these cells show a single band of hybridization at 4.5 kb. Amino acid starvation caused an increased abundance of this mRNA (Fig. 2). The time course of the effect was comparable to that observed for the transport activity, with mRNA abundance steadily increasing up to 16 h in this experiment. Proline and MeAIB, but neither glutamate nor arginine (all employed at a concentration of 2 mM), completely suppressed the stimulation of ATA2 expression.

The addition of 5 mM proline to amino acid-starved human fibroblasts had dramatic effects on ATA2 expression (Fig. 3). After a 3-h supplementation with the amino acid, ATA2

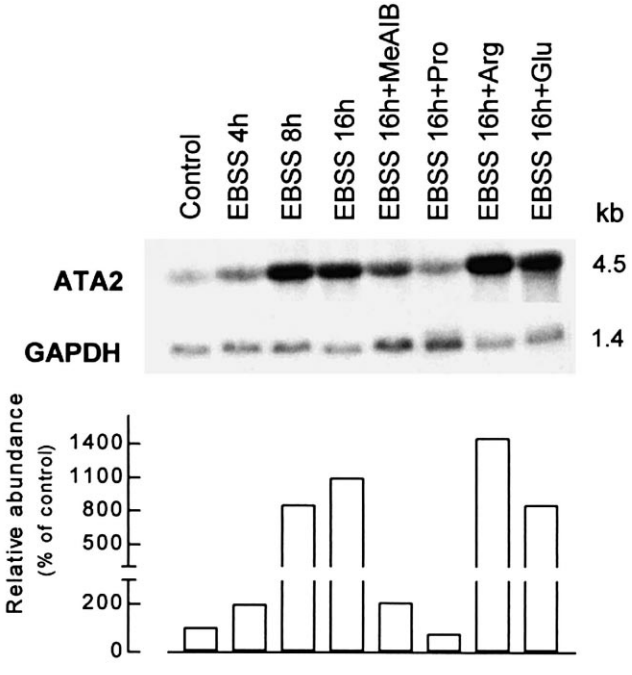

Fig. 2. Enhancement of ATA2 mRNA during amino acid starvation of cultured human fibroblasts. Upper: Northern analysis of ATA2 and GAPDH expression during incubation of cultured human fibroblasts in EBSS+FBS supplemented, where indicated, with selected amino acids employed at a concentration of 2 mM. Lower: The relative abundance of ATA2 mRNA was calculated according to Eq. 1. The experiment was repeated three times with comparable results.

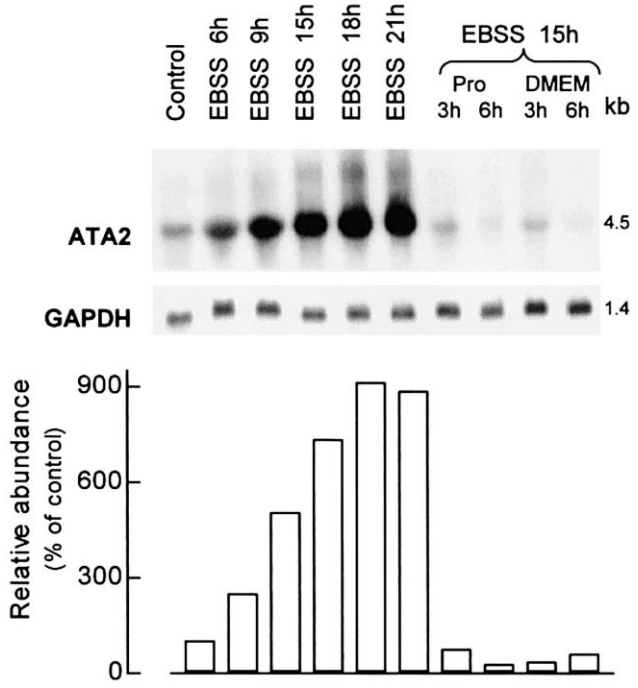

Fig. 3. Suppression of ATA2 overexpression by system A substrates. Upper: Cells were incubated for 21 h in EBSS+FBS. At 15 h fibroblast subcultures were transferred in EBSS supplemented with L-proline (5 mM) or in complete DMEM. RNA was extracted at the indicated times. Lower: The relative abundance of ATA2 mRNA was calculated according to Eq. 1. The experiment was repeated three times with comparable results.

mRNA levels decreased substantially at values comparable with control, unstimulated cells to become almost undetectable after 6 h. Comparable results were obtained if EBSS was replaced with complete DMEM.

4. Discussion

When cells are exposed to extracellular media lacking substrates of transport system A for neutral amino acids, the activity of the system slowly increases. Conversely, when extracellular substrate concentrations are restored, the transport activity falls to original values. The capability of system A to adapt its activity to the extracellular levels of the substrates was named 'adaptive regulation' and constitutes one of the main characteristics of this widespread transport mechanism. Described originally in avian fibroblasts [8], adaptive regulation has been described in all the cell models and tissues in which system A has been investigated. The main characteristics of this regulation are: (i) transport changes require hours for a full development; (ii) changes in transport activity depend upon protein synthesis; (iii) modulation of the transport capacity of the system (V_{max}) fully accounts for transport changes with the K_m substantially unaffected. These features suggested that adaptive regulation was to be referred to varying numbers of functional site A transporters present on the plasma membrane. The hypothesis that these changes are due to modifications in the expression of system A-related gene(s) was commonly accepted [6,16,17]. Two distinct phases of the regulatory mechanism were envisioned [6]: (i) a derepression phase, in which amino acid starvation would promote the synthesis of new site A transporters, thus accounting for the increase in transport activity; (ii) a repression phase, in which the addition of even a single substrate of system A causes a decrease in the transport activity, possibly referable to both a degradation of system A transporters and a block of transporter synthesis. This model was consistent with the results obtained with inhibitors of translation and transcription (see [7] for review). However, the failure of attempts to clone system A transporter gene(s) and/or to purify the transporter protein have thus far prevented the verification of this model.

In the last few months, the gene encoding a widespread transporter endowed with features typical of system A has been cloned [2–5]. This transporter, designated ATA2, is widely expressed in both epithelial and mesenchymal tissues. We show here that the abundance of ATA2 mRNA strictly parallels the transport activity of system A during both phases of adaptive regulation. In particular, amino acid starvation triggers a marked increase in ATA2 mRNA abundance that proceeds for at least 18 h (Fig. 3) when mRNA levels are nearly 10-fold enhanced compared to control. Stimulatory changes in both mRNA abundance and transport activity are completely suppressed by natural or analogue amino acid substrates of system A, such as L-proline and MeAIB. Moreover, the addition of these compounds to the extracellular medium of starved, high-transporting cultures produces a fall in ATA2 mRNA levels as well as, although more slowly, a decrease in the transport activity of the system. Interestingly, the substitution of the amino acid-free saline solution with a complete, amino acid-rich growth medium also suppressed ATA2 mRNA and produced an even faster decrease in transport activity of system A. These results are consistent with the hypothesis, suggested many years ago [6], that adaptive regulation acts at the level of gene expression, in both the derepression and the repression phase. However, the data presented here do not make it possible either to identify the site(s) at which gene expression is regulated or to assess the involvement of additional mechanisms acting at the protein level. Therefore, further investigations will be necessary to reach a complete understanding of this regulatory phenomenon.

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